

IONIZED CALCIUM AND CALCIUM-PROTEIN

BINDING IN BODY FLUIDS

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BINDING IN BODY FLUIDS

by

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This thesis is a summary of the following communications referred to in the text by their Roman numerals:

- I. Potentiometric determination of serum ionized calcium in a normal human population. Clinica Chimica Acta 40: 477, 1972.
- II. In vivo and in vitro studies on ionized versus total serum calcium in hyperparathyroidism. Acta Endocrinologica 74: 501, 1973.
- III. Electrophoresis-radioautography for the study of calcium-binding serum components. Clinica Chimica Acta 44: 67, 1973. In collaboration with J. Malmquist and O. Zettervall.
- IV. Human urinary protein with high calcium affinity. In collaboration with J. Malmquist, L. Tejler and O. Zettervall.
- V. Hypercalcemia and normal ionized serum calcium in a case of myelomatosis. Annals of Internal Medicine 78: 396, 1973. In collaboration with O. Zettervall.
- VI. Characterization of a calcium-binding IgG myeloma protein Scandinavian J. of Immunology, accepted for publication. In collaboration with O. Zettervall.

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INTRODUCTION AND PURPOSE

It is generally assumed that the main purpose of calcium homeostasis is to maintain a constant level of free calcium ions in the extracellular fluid. A substantial part of these calcium ions is bound to serum proteins, especially albumin. Proteins with a strong affinity for calcium ions have been isolated from some organs. It is probable that these proteins are involved in calcium metabolism. No such data are available about serum proteins.

The purpose of the present investigation was to analyse free calcium ions in serum and its relation to the total calcium concentration, and to find out whether the serum and the urine contain proteins with a strong affinity for calcium ions and if so, to elucidate their binding.

The various studies were designed

- (a) to define the normal range of variation of free calcium ions (Ca^{++}) in serum, and to evaluate the influence on Ca^{++} of age and sex, two factors of known importance in calcium metabolism.
- (b) to analyse statistically the Ca^{++} and the Ca_t (total calcium concentration) values obtained in patients with hyperparathyroidism and in a group of normocalcemic patients in order to find out whether Ca^{++} can discriminate borderline hypercalcemia better than Ca_t .
- (c) to relate Ca^{++} to Ca_t in two groups of hypercalcemic patients, one with hyperparathyroidism and one with myelomatosis, to detect individuals or groups where the Ca^{++} is normal or where the Ca^{++} is increased less than what might be expected from the Ca_t values.
- (d) to devise electrophoretic methods for identifying serum and urine proteins with a strong affinity for calcium ions.

BACKGROUND

CALCIUM HOMEOSTASIS

The total calcium content of the adult human body is estimated at about 1 kg (Mitchel et al. 1945). The skeleton contains 99 per cent of this amount (Pellegrino and Blitz 1965). The bone mineral consists of two calcium pools, a non-crystalline (amorphous) calcium salt and a crystalline (apatite) phase. About 35 per cent by weight of the mineral is amorphous calcium (Haper and Posner 1966). It is assumed that there is a continuous interchange between the calcium in extracellular fluid, about 1 g (Howard and Thomas 1963), and that in bone. Bauer et al. (1957) estimated that the exchangeable calcium amounted to about 5 g in adults. Neuman (1969) and Neuman and Ramp (1971) produced convincing evidence of the existence of a functional membrane separating the extracellular fluid of bone from the general extracellular fluids. The concentrations of calcium, magnesium and sodium are lower in bone fluids than in extracellular fluid whereas the concentration of potassium is high. The passage of calcium ions into and out of the skeleton is more than four times that due to resorption and remodeling (Neuman and Ramp 1971). It would therefore appear reasonable to assume that such a functional membrane must be partly responsible for the regulation of the serum calcium.

Serum calcium is considered to be one of Nature's most narrowly regulated physiological constants (McLean and Hastings 1935b). This constancy of the level of serum calcium seems to be necessary for many biological processes such as neuronal excitability, muscle contraction, membrane permeability, hormone release, enzyme activity and bone mineralization. Normally, blood plasma, considered as water, is supersaturated with respect to bone mineral. The physicochemical state of blood calcium is obviously therefore of importance.

Vitamin D, parathyroid hormone, and calcitonin play important roles in the overall calcium balance by their action on the kidney, intestine, and skeleton. Mathews et al. (1973) have proposed that these substances exert direct effect upon the passage of calcium ions through plasma membranes. However, it is not known exactly how serum calcium is regulated from moment to moment.

ASPECTS OF CALCIUM PROTEIN INTERRELATIONSHIPS

1. Calcified tissues

The states or forms in which calcium exists in extra-cellular fluids and its states when deposited in crystal form have been the subject of extensive investigation and debate.

A low serum calcium phosphate concentration product in rickets was first reported by Howland and Kramer (1922), and Shipley (1924) showed that rat cartilage calcifies in normal, but not in rachitic, serum. Holt et al. (1925) showed that normal adult plasma is supersaturated with respect to bone mineral. This finding has been confirmed by MacGregor and Nordin (1960) who found a solubility product for bone mineral, which was well below the presumed ion product in normal plasma. However, hydroxyapatite crystals do not form in an artificial solution of calcium and phosphate with the same concentrations as in serum, though added crystals grow at the expense of ions in the solution. Hence, the problem is to find out how the first aggregates of ions crystallize. It might occur by nucleation on a suitable component of an organic matrix.

Another important aspect of this problem is how crystal growth is controlled. One might presume that the head of the chemical pressure is always toward accretion on any already existing crystal. The existence of inhibitors of mineralization has therefore been assumed. Fleisch and Biaz (1962) thought that one of

these inhibitors is pyrophosphate. The glycoproteins of bone and dentine matrix, though present in only relatively small amounts, have recently attracted considerable interest, largely because of the part they may play in calcium binding (for review see Herring 1972, Weinstock 1972). Bone sialoprotein, isolated from bone matrix, has been found to be an efficient metal binder, as tested on yttrium, plutonium and americium. Furthermore, this glycoprotein, whose polypeptide chain contains more than 40% of acidic amino acids, has been found to bind calcium (Herring et al. 1971). In vitro sialoprotein inhibits calcium phosphate precipitation, but it is not known in what way this protein may take part in the control of mineralization in vivo (Herring 1972).

When bone matrix was digested with collagenase about 9.5 per cent remained as the non-collagenous fraction (Oldroyd and Herring 1967). To some extent the non-collagenous protein is made up of serum proteins. When ³H-glucosamine was injected into rabbits, it was found that of labelled material incorporated in the matrix, some was synthesized by bone cells, the remainder deriving from labelled serum glycoproteins (Owen and Shetlar 1968, Owen 1970). Furthermore, albumin has been extracted from bone tissue fluid and from calcified matrix, and in both cases shown to be immunologically identical with serum albumin (Owen 1973).

2. Urine

There is strong evidence for the existence, in urine, of inhibitors of spontaneous crystallization or of crystal growth. Thus, the addition of 1 per cent normal urine to a supersaturated solution of calcium oxalate markedly delays the growth and aggregation of crystals (Robertson et al. 1972). Treatment of rachitic rat cartilage with normal human urine does not result in any calcification (Thomas and Howard 1959), but while treatment with a corresponding artificial urine does (Lewis et al. 1966) Inorganic

pyrophosphate which forms a strong complex with calcium inhibited the mineralization only when it was added in much higher concentrations than those found in urine.

However, judging from the work of Howard et al. (1967) urine contains substances which are more potent than pyrophosphate in inhibiting mineralization of cartilage. An acidic peptide material with strong inhibitory potency was isolated from urine. This peptide was reported to be many times more potent than pyrophosphate in inhibiting cartilage mineralization and in preventing the formation of hydroxyapatite in vitro.

3. Biological transport systems

Cumulative evidence suggests that proteins are involved in calcium transport in different biological systems. Lately interest has also been focused on the active metabolites of vitamin D which play a central role in calcium homeostasis. It is now generally accepted that the dominant factor regulating calcium absorption from the intestinal tract is 1,25-dihydroxycholecalciferol. This renal steroid hormone acts via RNA in the induction of a specific calcium-binding transport protein (Emtage et al. 1973). The calcium-binding properties of this protein obtained from the chick have been investigated by Wasserman (1973). The molecule has at least two sets of calcium binding sites. The highest binding affinity of one set was calculated to about 2×10^6 l/M, which is to be compared with approx. 10^2 l/M for the albumin molecule (Pedersen 1971).

Other specific calcium binding proteins possibly involved in calcium transport have been isolated from the egg shell gland of the laying hen (Corradino et al. 1968), human renal tissue (Piazolo et al. 1971), and porcine parathyroid glands (Oldham et al. 1972).

4. Serum

Pribram (1871) was the first to suggest that part of the calcium in serum might be bound to protein. In 1911 Rona and Takahasi demonstrated the ability of proteins to form complexes with calcium ions.

The calcium-protein interaction was interpreted in quantitative terms by McLean and Hastings (1935a) by means of the frog heart method for measuring calcium ion activities. From empirical data it was possible to express the calcium-protein relationship according to the mass-law equation.

$$\frac{(\text{Ca}^{++}) (\text{Protein}^{--})}{(\text{Ca-protein})} = K$$

As the serum proteins were expected to vary in affinity for calcium with their amino acid composition and structure, the dissociation constant obtained for whole serum is a composite of the dissociation constant of the individual serum proteins. Strictly speaking, in the estimation of the ionized calcium fraction in clinical practice, the equation holds only when protein composition of the serum is normal.

Only few investigations of the protein-bound calcium fraction are available. This is partly because of the difficulties encountered in direct measurement of the calcium proteinate. The proteinate is usually measured indirectly by determining the total calcium and the ultrafilterable calcium. The non-diffusible part is believed to be calcium protein chelates. The other possibility is to isolate individual plasma proteins and study the affinity between them and calcium ions in vitro. Moore (1969) and Pedersen (1971a) have produced evidence that serum albumin accounts for 80-90 per cent of the calcium binding capacity of proteins in normal serum. But nothing suggests that this calcium binding to albumin, has an independent role in calcium homeostasis. Rather, it

would appear plausible that the binding of the calcium ions to the albumin only reflects the prevailing concentrations of free calcium ions and albumin molecules.

There are only a few observations indicating the existence of serum macromolecules other than albumin with the same or even greater affinity for calcium ions. On statistical analysis of total calcium, albumin, and two globulin fractions in 128 cases with various protein concentrations and in sera with, by definition, normal ionized calcium Gutman and Gutman (1937) found a calcium fraction, presumably bound to a globulin fraction, whose concentration was not affected by increases in total globulin content. It was therefore inferred that this globulin bound more calcium per gram than albumin. Moore (1969) found that the calculated percentage of calcium albuminate in patients with cancer and associated hypercalcemia was abnormally low, while calcium globulinate accounted for 33.5 per cent of the calcium proteinate, which is significantly higher than in normals. In five patients with the nephrotic syndrome Pedersen (1972b) found the average calcium binding affinity to be normal in spite of the fact that all the patients were severely hypoalbuminemic.

Isolated polyclonal gamma globulin shows only questionable calcium binding (V). The ability of myeloma proteins to bind calcium has been widely studied (Rawson and Sunderman 1948, Prasad and Flink 1950, Glueck et al. 1960, Van Leeuwen 1964) but with conflicting results. The discrepancies may be due partly to the use of inappropriate methods.

RESULTS AND DISCUSSION

DETERMINATION OF SERUM IONIZED CALCIUM (I, II, V)

Measurement of calcium ions in plasma or serum, which had long been desirable because of the many biological processes influenced by the calcium ion activity, was not possible until 1934, when McLean and Hastings devised the classical bio-assay technic for estimating serum ionized calcium from its effect on contractions of the isolated frog heart. Their method is very time-consuming and has not been widely used. In 1951 Raaflaub introduced a chemical method for determining ionized calcium in protein-free fluid with the indicator murexide. But neither has this spectrophotometric method been widely used because it is difficult to obtain protein-free blood samples and, second, because neither the precision nor the sensitivity of the method is really acceptable. However, by using an improved ultrafiltration method and tetramethylmurexide as an indicator Pedersen (1970a, 1970b) reported very good reproducibility of the Ca^{++} values measured with this technic.

Endeavours to measure the calcium ion activity in biological fluids with electrodes had previously been unsuccessful because, among other things, the electrodes were rapidly poisoned by small amounts of proteins. In 1967, however, Ross devised a calcium selective electrode, which permitted measurement of the calcium ion activity in small volumes of protein-containing fluids under anaerobic conditions. The principles of the so-called ion selective electrodes have been described by Moore (1968). In the calcium sensitive electrode a linear relation exists between the measuring potential developed by the electrode and the logarithm of the calcium ion activity in the solution examined. The ionic activity is a product of the calcium concentration in the solution and the activity coefficient of the calcium. This activity coefficient varies with the ionic strength of the solution, and

therefore it is important that the standard solutions used for calibration of the electrode have the same ionic strength as the fluid, whose calcium ion activity is to be measured. The activity coefficient of a standard solution cannot be accurately measured, but is assumed to be equal to that in serum provided the ionic strength is the same in both. In the present study the calcium ion activity in serum is given as the concentration of free calcium ions (Ca^{++}), i.e. related to CaCl_2 -NaCl solutions with known calcium concentrations and with a total ionic strength close to the expected ionic strength of serum, i.e. about 0.16 M. The present flow-through calcium electrode has a sodium error that affects measured Ca^{++} by only about 4 per cent when the sodium ion concentration differs by 28 mmol/liter from the normal standard value (Ladenson and Bowers 1973a). Therefore, only in patients with severe electrolyte disturbance is any systematic error to be expected.

The practical use of the electrode in routine work offers certain difficulties because of electrostatic disturbances and the drift of the electrode. Therefore, in order to be sure that the electrode gave stable measuring values (I) it was considered necessary to check every measurement of serum with a standard solution both before and after testing of the serum. On the other hand, the difference in measuring potential between two different standard solutions rarely varies in the course of a working day. Certain factors capable of severely affecting the results of measurement received special attention.

1. Preparation of patients and blood samples

In a previous study (Lindgärde and Zettervall 1971) it was shown that Ca^{++} was not influenced by change in posture. This finding has been confirmed by other authors (Pedersen 1972a, Husdan et al. 1973). However, the well-known changes in Ca_t and serum proteins following alterations in posture are considered to be entirely consistent with this mechanism

(Husdan et al. 1973). Therefore, in this study, the blood collection was performed with the subjects sitting (I) or lying (II, V). Venous blood was allowed to flow into glass tubes containing oil. It was found that a brief exposure of whole blood to air did not demonstrably alter the calcium ion activity. Similar data have been presented by Radde et al. (1971). Storage of serum for up to five hours at room temperature did not affect the Ca^{++} activity. If sera were stored at 4°C for 24 h there was likewise no appreciable change in calcium ion activity (Lindgärde and Zettervall 1971).

2. Ca^{++} standards

The presence of trypsin and triethanolamine in the standard solutions stabilizes the potential readings. However, this addition has been shown to gradually alter the apparent Ca^{++} concentrations of the standards. This effect has been shown to be due to triethanolamine only for the first 4 hrs storage at room temperature (I). Burr (1973) has found that a variety of organic bases, including triethanolamine, interfered with the function of the present calcium-specific electrode. Therefore only non-buffered standards have been used for calibration, but trypsin has been utilized for rinsing the system after each sample.

3. The influence of pH on Ca^{++} activity

The mean serum pH values for different groups reported in this study varied between 7.38 and 7.41. The mean pH values for fresh sera given by other authors have varied between 7.35 (Fuchs 1972) and 7.47 (Hattner 1970). These differences may in part explain the variability in mean Ca^{++} levels presented in literature. A stable pH level is very important for estimating Ca^{++} . The hydronium and calcium ions seem to compete for the same binding sites on

the proteins. The effect can be shown in vitro by adding increasing amounts of HCl or NaOH to pooled normal serum, giving a pH range from 6.70 - 8.53. If the Ca^{++} and pH values are plotted against each other, semilogarithmically, a straight line with a slope of - 0.297 is obtained. This slope is the same as Schwartz et al. (1971) demonstrated (-0.30) using a gas equilibrium system to get the pH range 7.10 to 7.7. The relationship between Ca^{++} and pH can be expressed as: $\log \text{Ca}^{++} \text{ at pH } 7.38 = \log \text{Ca}^{++} \text{ at pH measured} + 0.297 (\text{pH } 7.38 - \text{pH measured})$. The competition of H^+ and Ca^{++} for protein binding sites in vivo was demonstrated in a pilot study. In a normal individual hyperventilation for 25 min. decreased Ca^{++} and increased pH and Ca_t . However, the Ca^{++} decrease was less than that calculated from in vitro data, and this finding may explain the Ca_t increase. Seamonds et al. (1972) have reported that respiratory alkalosis caused a decrease in Ca^{++} of 0.10 mEq/l per 0.1 unit increase in pH. In contrast, the metabolic alkalosis induced by ingestion of food caused a decrease in Ca^{++} of 0.21 mEq/l per 0.1 unit increase in pH. In the present study the Ca^{++} decrease was 0.10 mEq/l per 0.1 pH unit, i.e., the same result as Seamonds et al. have presented.

4. Variation of Ca^{++} in a normal population with respect to age and sex

In normal individuals, variation in Ca_t has been proposed to be due to variations in serum albumin levels. In normal adults (576 persons) and in 2005 unselected ambulant patients Keating et al. (1969) found that Ca_t decreased with increasing age in men, but did not change significantly in women, i.e., the same pattern as reported in the present study. Keating therefore suggested that diffusible calcium may decrease in men and increase in women. The data presented here do not lend support to this theory as Ca^{++} decreases with age in both sexes ($p < 0.001$ by linear regression analysis).

The same low level of Ca^{++} in old subjects (70 - 96 years) was found in both sexes, and as about 90 per cent of diffusible calcium is ionized, it is reasonable to assume that the diffusible fraction decreases not only in men but also in women.

In young individuals (19 - 45 years) the mean Ca^{++} value was higher for men than for women ($p < 0.025$). The same difference has been found by Ladenson and Bowers (1973a). Statistical analysis of the means of Ca^{++} in the age groups, not divided according to sex, showed that no notable difference occurred in Ca^{++} before the age of 60 years.

The same tendency with age has been found for intestinal calcium absorption. Bullamore et al. (1970) found a significant decrease in absorption of calcium in both men and women at the age of 80.

5. The relation between Ca^{++} - Ca_t

The wider the range of variation of Ca_t in a population the higher the significance of the correlation between Ca_t and Ca^{++} . In hyperparathyroidism the coefficient of correlation was found to be high (0.85, $p < 0.001$). The coefficient was not so high in a normal population 0.57 ($p < 0.001$) for men and 0.43 ($p < 0.001$) for women. This means that in the normal range of variation of Ca^{++} the values found for Ca_t are, as expected, markedly influenced by other factors, particularly the concentration of albumin (Moore 1969).

SERUM CALCIUM VALUES IN HYPERPARATHYROIDISM (II)

The aim of this study was to evaluate the usefulness of Ca^{++} determination in borderline hypercalcemia and to study

the relationship between Ca^{++} and Ca_t in order to get information about the calcium-binding capacity in hyperparathyroid patients. The relation between ionized and protein-bound calcium is governed by physical and chemical factors. The most important factors are concentrations of protein and calcium, temperature, pH, and ionic strength. Since the ionic strength and temperature are considered to be constant and the pH influence is eliminated by the use of the correction formula given earlier, the problem narrows down to determination of Ca^{++} , Ca_t , and protein.

It is of interest to compare the values for ionized calcium that are calculated from total protein and total calcium concentration with those observed with the electrode. Harris and De Mets (1971) have estimated the Ca^{++} level using the McLean-Hastings equation. From each of 68 normal subjects one sample a week was obtained for 10 - 12 weeks. The mean total calcium and total serum protein were 5.10 mEq/l and 6.96 g/100 ml, respectively. Assuming an albumin/globulin ratio of 1.8 (Van Slyke 1929) and using the concurrently observed albumin/globulin ratio (mean 1.57) the calculated Ca^{++} values were 2.34 and 2.36 mEq/l, respectively. In the present control group the corresponding average concentrations were Ca_t 4.86 mEq/l, total serum protein 6.92 g/100 ml and Ca^{++} 2.30 mEq/l. The McLean-Hastings equation assumes a pH value of 7.35. Therefore, if the estimated Ca^{++} values are calculated according to the presented pH- Ca^{++} correction formula, Ca^{++} decreases to 2.29 and 2.31 mEq/l, i.e., the same value as in the control group. According to Harris and De Mets, a three-fold change in albumin/globulin ratio changes the estimated Ca^{++} only by 5 per cent when Ca_t is within the physiological range.

However, it seems reasonable to assume an interindividual variation of the calcium-binding capacity that cannot be measured by protein determination alone. This theory was

partly substantiated when increasing amounts of calcium chloride were added to eight individual or pooled normal sera in vitro at constant protein concentration. The correlation coefficients for Ca^{++} and Ca_t were 0.99 - 1.00 in all cases except one (linear regression analysis). The slope of the regression line varied from 0.50 - 0.68 indicating variation in the capacity of the sera to bind calcium ions.

As the protein concentrations in the control and in the hyperparathyroid groups were the same, the Ca^{++} and Ca_t data were studied statistically. A co-variance analysis of the two groups suggested that the control values fell outside the regression line for the data of the hyperparathyroid group. Hence, the relationship between Ca^{++} and Ca_t in vivo might be better described as curvilinear than as linear. This was confirmed by the fact that a regression curve consisting of two straight lines meeting at an angle would describe the data more accurately than a single line ($p = 0.008$). The point at which the two lines met was found at $\text{Ca}_t = 5.55 \text{ mEq/l}$, $\text{Ca}^{++} = 2.75 \text{ mEq/l}$. Up to the meeting point Ca^{++} increased more than Ca_t as the slope of the regression line was 0.628. This means that at apparently normal protein concentration Ca^{++} determinations may disclose borderline hypercalcemia better than Ca_t . This finding confirms the original observation by Lloyd and Rose (1958) regarding the usefulness of estimation of Ca^{++} in hyperparathyroidism.

This, however, does not corroborate the assumption by Lloyd and Rose that the parathyroid hormone reduces the number of serum ligands with calcium-binding capacity or affinity of the ligands. Since Ca^{++} increased more than Ca_t in vitro on addition of CaCl_2 , it is reasonable to assume that when the same observation was made in vivo the relative Ca^{++} increase compared with that of Ca_t was only an effect of the law of mass action in the prevailing calcium and protein concentrations.

Strangely enough, the slope of the regression line decreased with increasing calcium concentration. Nothing suggests that the ligands of the complexed calcium fractions, e.g. phosphate and citrate, increase in concentration in hyperparathyroidism (Walser 1962, Wills 1972). The protein concentration did not differ from that in the control group and did not tend to increase with increasing Ca_t . It is therefore reasonable to assume that the observed change between Ca^{++} and Ca_t was due to the changed concentration or affinity of the protein ligands.

ELECTROPHORETIC STUDIES OF CALCIUM-BINDING TO SERUM COMPONENTS (III)

Most of the protein-bound calcium fraction consists of an albumin chelate. Albumin-calcium interaction has been studied intensely (Pedersen 1971). On the other hand, it is not known which proteins are responsible for the remaining calcium-binding. Neither is anything known about the prevailing affinities. The purpose of the present study was to devise methods capable of demonstrating all major calcium binders in serum after electrophoretic separation.

With the aid of radioautography it was shown that under the conditions described ^{45}Ca ions migrated regularly and in a compact group at a rate of 3 mm/min through agarose gel towards the cathode. When ^{45}Ca was mixed with serum just before application to the gel plate, ^{45}Ca migrated, as expected, in the opposite direction towards the main part of the proteins and at the site of the proteins the radioautogram was blank. On the other hand, when the buffer system contained ^{45}Ca and when calcium ions were allowed to meet the separated proteins, the radioautogram showed a certain accumulation of ^{45}Ca at the place of the albumin.

This interaction between calcium and albumin was illustrated better by two-dimensional electrophoresis. Immediately after

the end of serum electrophoresis (55 min) the gel plate was turned 90°. A well, 5 x 80 mm, cut at right angles to the site of application and at a distance of 10 mm from it was filled with agarose solution containing ^{45}Ca . The current was then applied for 12 minutes, directing polarity to make the ^{45}Ca ions traverse the separated serum proteins. An interaction between serum albumin and ^{45}Ca was seen as a local deviation of the radioautographic band depicting the position of ^{45}Ca ions after electrophoretic migration across the serum proteins. No such interaction was seen in other serum protein regions. This disturbance of radiocalcium migration was found to vary with the albumin concentration as well as with the calcium concentration in the buffers. Addition of calcium to the buffer diminished the extent of albumin ^{45}Ca interaction.

Dilution of serum with 0.15 M NaCl and sera from hypoalbuminemic patients resulted in a minor deviation of the radio-calcium band at the site of the albumin. It would therefore appear that the demonstrated interaction between calcium and albumin was due to the affinity between albumin and calcium ions.

Another possible explanation, at least theoretically, is that the ^{45}Ca -ions are prevented non-specifically by the albumin molecules. Sera from patients with multiple myeloma and M-components in varying concentrations of 1.4 to 6.8 g/100 ml were therefore examined. In only one case, patient A.L., was an interaction found between ^{45}Ca and the M-component. This particular M-component has a specific affinity for calcium (V). Therefore, rapid association and dissociation would seem to be an adequate explanation for the pattern observed with albumin in the two-dimensional method. Apart from the interaction between albumin and calcium the two-dimensional electrophoretic method does not teach anything about the calcium binding properties of the proteins occurring in normal serum. This

may be due to the method not being sensitive enough at low protein concentrations or when the affinity for calcium ions is low. Since one of the purposes of the investigation was to find out whether the serum contained proteins with a high affinity for calcium it was considered desirable to test the following hypothesis.

The binding of calcium ions to albumin is believed to be momentary. One might assume that the higher the affinity between a protein and the calcium ions, the more of the calcium-binding groups are taken up by calcium ions when the system is in equilibrium, i.e. in a serum sample in vitro. Since only a relatively small number of radioactive ions are added to the serum in vitro it will probably take a long time for a certain part of the ^{45}Ca ions to be bound to these hypothetical proteins by an exchange reaction. The binding of ^{45}Ca to such proteins must therefore be slow.

The following experiment was therefore performed to find out whether the autoradiographic findings varied with the duration of the incubation time. One day after ^{45}Ca had been added to the serum, which was afterwards stored at 4°C before it was examined electrophoretically, the radioautogram showed two bands in the α_2 and β - regions, respectively. The increasing intensity of the blackening was noticed even after 3 days' incubation of the serum sample before electrophoresis. ^{45}Ca was thus slowly bound to these proteins. These bands were found in all of the patients and normal persons examined.

The nature of the serum components is unknown, but they are not of low molecular weight because they can be visualized after dialysis for three days against 0.15 M NaCl with repeated exchange of the dialysis fluid (unpublished observations).

IDENTIFICATION OF A URINARY PROTEIN WITH HIGH CALCIUM AFFINITY (IV)

By use of the one-dimensional electrophoresis-radioautography method (III) it was possible to visualize a calcium-binding component with α_2 mobility in concentrated human urine. The radioautographic band was demonstrated in urines from normal individuals as well as from patients with various diseases. In some of the urine concentrates, a protein band, corresponding in location to the radioautogram band, was clearly visible on the stained electrophoresis plate.

After proteolytic digestion with trypsin the calcium-binding capacity and the uptake of protein stain were considerably attenuated. Accordingly, the urinary calcium binder is considered to be a protein.

On gel filtration on Sephadex G-100 the protein (detected by electrophoresis-radioautography of effluent fractions) emerged between bovine serum albumin and cytochrome c, at a position almost coinciding with that of ovalbumin (MW 45,000). Calculations based on V_e/V_o values indicated a molecular size corresponding to a molecular weight of about 42,000 daltons.

In some experiments the urine concentrates were dialysed against Tris-buffered saline, or against the same solution containing EDTA before ^{45}Ca addition. Serial electrophoresis-radioautograms revealed that such dialysis increased the rate of isotope uptake, as well as the amount of ^{45}Ca finally bound. This increased uptake was most pronounced after EDTA dialysis. The findings suggest that the number of sites available for binding of ^{45}Ca had been increased by dissociation of previously bound calcium.

The affinity of the protein for calcium seems to be very high. This was demonstrated by competitive binding studies. Thus, after simultaneous addition of ^{45}Ca and a large (83-fold) molar excess of EDTA, the radioautogram still showed isotope binding to the protein although to lesser extent.

In urines from three patients with hypercalciuria, electrophoresis-radioautograms showed larger amounts of ^{45}Ca being bound than in normal urines. Accordingly, larger amounts of the calcium-binding protein would seem to be present in the pathological urines, as also evidenced by protein staining. Understanding of the relation between this finding and the hypercalciuric state requires further studies.

HYPERCALCEMIA IN MYELOMATOSIS (III, V, VI)

Ca^{++} and Ca_t were determined in 15 patients with a firm diagnosis of myelomatosis and hypercalcemia.

Sera from 12 of the patients contained a monoclonal immunoglobulin band (M-component). The concentration of this fraction varied between 1.4 and 6.8 g/100 ml. In 2 patients electrophoresis showed no M-component, but light chains (Bence-Jones protein) in the serum and urine, and in one patient only light chains in the urine.

1. Measurement of Ca^{++} and Ca_t

Ca^{++} was measured in 4 patients with trypsin and triethanolamine in the standard solutions according to a method described by Lindgärde and Zettervall (1971). The Ca^{++} determination in the other 11 patients were made according to the present method (I). The normal range was defined as the mean ± 2 SD of respective population studies. In all the patients except A.L. the Ca^{++} was increased on one or more occasions. In 6 of the patients the Ca_t was normal on at least one occasion

when Ca^{++} was increased. The most probable explanation of this discrepancy is a tendency to low albumin values and/or low pH-values in renal disease. In one patient with albumin values in the lower part of the normal range, normal renal function and pronounced hypercalciuria, Ca^{++} was slightly increased when Ca_t was normal. The patient's diet was regulated in respect of calcium content (800 mg/day). This finding may be interpreted in the same way as the results obtained in the investigation on hyperparathyroidism (II), namely that Ca^{++} discriminates borderline hypercalcemia better than Ca_t .

2. Influence of pH

Acute hypercalcemia, particularly in patients with neoplastic disease, has been observed to be associated with metabolic alkalosis (Heinemann 1965). Alkalosis in myelomatosis is of much shorter duration than hypercalcemia (unpublished data). Alkalosis (standard bicarbonate > 26 mM) was noted in 6 of the present patients.

A retrospective study of hypercalcemia in another series of patients with myelomatosis revealed alkalosis in 8 of the 37 hypercalcemic ($\text{Ca}_t > 5.4$ mEq/l) patients. In 10 patients where Ca_t was more than 5.4 mEq/l on at least 2 occasions statistical analysis of 47 simultaneous Ca_t and standard bicarbonate showed a clear correlation ($r = 0.66$ $p < 0.001$; linear regression analysis) (unpublished observations).

In the investigation of the aetiology of hypercalcemia in myelomatosis it is important to correlate the Ca^{++} and Ca_t values with the serum pH. Ladensen and Bowers (1973b) described a patient with myeloma with normal $\text{Ca}^{++} = 2.65$ mEq/l (normal range; 2.25 - 2.75) and a corresponding $\text{Ca}_t = 5.55$ mEq/l (normal range; 4.55 - 5.15) and pH 7.53 (normal range 7.38 - 7.45). The authors suspected that the patient had a calcium-

binding paraprotein. But when Ca^{++} was replaced by a Ca^{++} value corresponding to pH 7.38 according to the previously described correction formula (I), Ca^{++} was 2.94 mEq/l, i.e. a clearly elevated value.

Similar values have been observed in 1 of the 6 alkalotic patients in the present investigation. On one day during a spell of alkalotic hypercalcemia one patient (A.R.) was found to have the following values:

$\text{Ca}^{++} = 2.56 \text{ mEq/l}$ (normal range; 1.95 - 2.51)

$\text{Ca}_t = 6.6 \text{ mEq/l}$, pH/s 7.62. When the alkalosis disappeared in vivo Ca^{++} rose to values corresponding to Ca_t . Likewise the M-component in A.R. showed no calcium-binding capacity on electrophoretic examination (III).

3. Hypercalcemia and normal ionized calcium in a case of myelomatosis

A repeatedly normal serum Ca^{++} level despite Ca_t values of 6.1 to 7.2 mEq/l (normal range: 4.9 ± 0.4 (2 SD)) in one patient (A.L.) strongly suggested that the M-component (type IgG1; concentration about 6.5 g/100 ml) bound calcium. The clinical diagnosis was later confirmed by roentgenographic demonstration of typical skeletal lesions. During continuous hypercalcemia (observation period 2 years) A.L. showed no tendency to alkalosis or clinical evidence of hypercalcemia, and the excretion of calcium in the urine was normal.

In order to confirm the calcium-binding property of the M-component the M-component was isolated in the following way. After ion exchange chromatography in DEAE-cellulose and gel filtration in Sephadex G-200 pure polyclonal gamma-globulin and a preparation of M-component with only traces of polyclonal gammaglobulin were obtained from serum of patient A.L. With ion exchange chromatography M-components were prepared in the same way from serum from two other

patients with hypercalcemia and myelomatosis.

The calcium-binding capacity of these protein preparations and pooled normal gamma globulin (Kabi) was studied by equilibrium dialysis. Plotting of the bound calcium (c.p.m. bound ^{45}Ca) against the protein concentration of the M-component from A.L. showed a linear correlation. The polyclonal gamma globulin from A.L., and the other two M-components showed only questionable calcium binding compared with that of the patient's M-component.

4. Characterization of the calcium-binding M-component in the patient A.L.

The M-component from A.L. is the first known myeloma globulin to bind calcium. Since it is an immunoglobulin it was considered of interest to try to determine the number of calcium-binding sites and to assign the calcium bonds to known subunits of the immunoglobulin molecule.

Conventional equilibrium dialysis where the protein concentration is constant and the calcium concentration varies was performed. The results obtained were afterwards used for a Scatchard plot. The plot showed a linear relation and the number of binding sites were calculated as 2.4 per Ig-molecule. These results suggest that the two calcium-binding sites are identical and independent of one another.

The calculated apparent intrinsic association constant, k , was 10^4 l/M. This affinity for calcium ions is within the range of those found for myeloma proteins with antihapten activity (Eisen et al. 1967, 1970).

Equilibrium dialysis with constant calcium concentration and varying protein concentration to compare the calcium-binding between papain fragment from the M-component and

undigested M-component showed that at the same protein concentration the Fab preparation bound more ^{45}Ca than the undigested M-component. Assuming that the molecular weight of a Fab fragment is one third of that of the M-component, the ratio between the bound calcium and the calcium bound to Fab and the M-component should be 1.5. The actual ratio found from the bound calcium versus the protein concentration regression lines was 1.3. The reasonable agreement with the theoretical ratio suggests that the association constant of the Fab fragment and that of the M-component are compatible.

Since practically all of the Fc material was precipitated during dialysis, no reliable value was obtained for this preparation. However, the experiment with two-dimensional electrophoresis-radioautography showed that calcium-binding activity was correlated with the Fab, but not with the Fc fragment. It was therefore concluded that the calcium-binding sites of the M-component are situated within the Fab region. Thus, the bivalency of the M-component, as suggested by the result of the Scatchard plot analysis, is confirmed, since the IgG molecule contains two Fab regions.

After reduction, alkylation and lowering of pH heavy and light chains of the M-component were separated. Equilibrium dialysis showed that the reduced and alkylated, but not dissociated, M-component binds calcium, but to a less extent than unreduced M-component.

Calcium-binding activity was also found in the light chain. On the other hand, only insignificant calcium-binding activity was found in the free, heavy chain preparation. Since the concentration of this preparation was low it was difficult to evaluate the finding. When heavy and light chains were allowed to recombine it was also possible to demonstrate calcium binding but it is uncertain whether the recombined molecules in the mixture really bound calcium.

On a weight basis light chains were poorer calcium binders than reduced and alkylated M-component. Theoretically, several suggestions may be offered to explain this difference. One possible explanation is that in the intact molecule the configuration of the calcium-binding site is influenced by the heavy chain and thereby the affinity is potentiated. Isolated light and heavy chains normally lose the major part of the antigen-binding capacity of the native molecule. It has nevertheless been reported that both light (Goodman and Donch 1965, Yoo et al. 1967) and heavy chains (Fleischman et al. 1963, Utsumi and Karush 1964, Haber and Richards 1966) alone can bind an antigen. Hence, the fact that at least part of the calcium-binding configuration is situated in the light chain does not rule out the possibility of calcium being bound in a genuine antigen combining site.

On comparison by electrophoresis the monoclonal light chains migrated faster toward the anode than did the bulk of light chains from a polyclonal IgG preparation. The calcium-binding activity in A.L.'s light chains was demonstrated with two-dimensional electrophoresis in both separated light chains and Bence-Jones protein. But the calcium-binding properties of the light chains were apparently not due solely to a high negative net charge because other Bence-Jones proteins with still higher electronegative charges did not bind calcium with certainty (unpublished electrophoresis-radioautography observations). It may therefore be tentatively concluded that the appreciable calcium ion affinity of this particular light chain is due to a suitable sterical arrangement of several negatively charged groups.

SUMMARY AND CONCLUSIONS

The present investigation deals with two aspects of calcium in human serum. Firstly, the calcium ion activity has been determined by the use of a calcium-specific electrode. Secondly, proteins with high calcium affinity have been detected. The method used for the latter purpose also allowed the demonstration of a urinary calcium binding protein. This protein has been partially characterized.

The following conclusions were made as regards free calcium ions:

- (a) The mean serum ionized calcium in 101 normal individuals aged 19-96 was 2.23 ± 0.14 (1.S.D.) mEq/l.
- (b) A significant decrease in ionized calcium with age was observed in both sexes. In young individuals the mean value was higher for men than for women.
- (c) Statistical analysis showed that determination of the ionized calcium may be superior to total calcium measurement of disclosing borderline hypercalcemia as a means in patients with suspected primary hyperparathyroidism.
- (d) In hypercalcemia due to hyperparathyroidism the fractional increase of ionized serum calcium was somewhat lower than that of total calcium. This would seem to indicate that an increased calcium binding capacity exists in serum in this disease.
- (e) Among 15 patients with myelomatosis and elevated serum calcium one patient with normal ionized calcium was found. The myeloma protein (M-component) of this patient was found to be a strong calcium binder.

The following conclusions were made with respect to proteins with high affinity for calcium ions.

- (a) With the aid of radioautography, the association of ^{45}Ca with electrophoretically separated serum components was studied. Calcium was found to bind to albumin and to the components in the α_2 and β regions. The affinity of the latter calcium binders seemed to be high.
- (b) By use of the electrophoresis-radioautography method it was possible to identify a urinary protein with high calcium affinity.
- (c) A calcium binding myeloma globulin was isolated. The protein was shown to have two independent binding sites for calcium ions. The sites were localized in the Fab regions and isolated light chains demonstrated calcium binding activity.

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